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The PAF Complex Regulation of *Prmt5* Facilitates the Progression and Maintenance of MLL-Fusion Leukemia

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Abstract

Acute myeloid leukemia (AML) is a disease associated with epigenetic dysregulation. 11q23 translocations involving the H3K4 methyltransferase *MLL1* (*KMT2A*) generate oncogenic fusion proteins with deregulated transcriptional potential. The Polymerase Associated Factor complex (PAFc) is an epigenetic co-activator complex that makes direct contact with MLL fusion proteins and is involved in AML, however its functions are not well understood. Here, we explored the transcriptional targets regulated by the PAFc that facilitate leukemia by performing RNA-sequencing after conditional loss of the PAFc subunit Cdc73. We found Cdc73 promotes expression of an early hematopoietic progenitor gene program that prevents differentiation. Among the target genes, we confirmed the protein arginine methyltransferase *Prmt5* is a direct target that is positively regulated by a transcriptional unit that includes the PAFc, MLL1, HOXA9 and STAT5 in leukemic cells. We observed reduced PRMT5-mediated H4R3me2s following excision of *Cdc73* placing this histone modification downstream of the PAFc and revealing a novel mechanism between the PAFc and Prmt5. Knock down or pharmacologic inhibition of *Prmt5* causes a G1 arrest and reduced proliferation resulting in extended leukemic disease latency *in vivo*. Overall, we demonstrate the PAFc regulates Prmt5 to facilitate leukemic progression and is a potential therapeutic target for AMLs.

Keywords

Leukemia; MLL; Epigenetics; PAFc; PRMT5; small molecule inhibitors

Introduction

Dysregulation of transcriptional and epigenetic mechanisms are increasingly recognized as critical events in the development of both acute myeloid (AML) and acute lymphoid leukemia (ALL). Over 70% of AML patients are estimated to harbor mutations in epigenetic

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regulatory proteins[1]. The Polymerase Associated Factor complex (PAFc) is an epigenetic co-activator protein complex that is essential for the proliferation of various forms of AML[2-4], including those harboring *MLL1* translocations. The PAFc is also linked to solid tumors where subunits are mutated or overexpressed in various cell types, suggesting context dependent functions[5-8]. The PAFc is composed of six subunits consisting of CDC73, PAF1, CTR9, LEO1, RTF1 and SKI8 in human cells[9-11]. Through the CDC73 subunit, the PAFc associates with the CTD of RNA Pol II facilitating transcriptional initiation, elongation, and termination[12, 13]. Once localized to targets, the PAFc helps recruit transcriptional and histone modifying complexes associated with transcriptional activation[14-17]. For example, BRE1 of the E2/E3 ubiquitin ligase complex RAD6/BRE1, which catalyzes mono-ubiquitination of histone H2BK120, directly binds to the PAF1 subunit of the PAFc[18]. Additionally, the Rtf1 subunit was recently described to directly interact with Rad6 to regulate H2B ubiquitylation[19]. Further, we and others have shown that the PAF1 and CTR9 subunits make direct contact with the H3K4 methyltransferase Mixed Lineage Leukemia 1 (MLL1)[2, 4, 20].

MLL1 is involved in chromosomal translocations to one of over 70 fusion partners resulting in expression of oncogenic MLL fusion proteins[21]. In adult AML and ALL, around 10% of patients present with MLL-translocations, which increases to 50% for infant AMLs[22]. Interestingly, while the PAFc-MLL1 interaction is essential for the proliferation of several subtypes of AML (including those with MLL translocations), disruption of the PAFc-MLL1 interaction is tolerated in hematopoietic stem cells, thus identifying the PAFc as a potential therapeutic target[3, 4]. In addition to leukemia, various subunits of the PAFc have been implicated in a variety of solid tumors. For example, *PAF1* is overexpressed in pancreatic cancer[7]. While, germline mutations in the *CTR9* subunit have been described in Wilms tumor[6], overexpressed CTR9 correlates with a poor prognosis through increased transcriptional activation in ERα+ breast cancer[8]. The CDC73 subunit coded by the *HRPT2* gene, is commonly mutated in hyperparathyroidism-jaw tumors pointing to a tumor suppressor function[5]. However, CDC73 is overexpressed in liver and breast cancer[23]. These data point to context dependent functions for subunits of the PAFc to act as oncogenes and tumor suppressors. In leukemic cells, our data demonstrated the PAFc is necessary for MLL1 recruitment to and activation of *Hoxa9* and *Meis1*, which are thought to act as oncogenic drivers in as much as 50% of human AML[24]. Despite a clear requirement for the PAFc in leukemic cells, the transcriptional programs controlled by the PAFc remain unclear.

Here, we investigated the gene expression programs controlled by the PAFc in AML cells harboring MLL fusion proteins. We discovered the PAFc promotes expression of a subset of pro-leukemic genes associated with self-renewal and a histone methyltransferase program. Within the latter program, we identified protein arginine methyltransferase 5 (*Prmt5*), amongst several *Prmts*, as a direct transcriptional target of the PAFc. PRMT5 deposits symmetric methyl marks on histone residues H4R3, H3R2, and H3R8[25-27] and is overexpressed in several solid cancers[28, 29]. As such, clinical trials are underway testing small molecules designed to inhibit PRMT5 enzymatic activity in patients with relapsed solid tumors and non-Hodgkin's lymphoma (ClinicalTrials.gov Identifier: NCT02783300). Still, conditional loss of *Prmt5* in the hematopoietic system is lethal due to defective HSC cycling underscoring a need to better understand the regulation and function of Prmt5 [30].

Investigating the role of Prmt5 in AML, we discovered the PAFc along with STAT5, MLL1, and HOXA9, bind to the *Prmt5* locus and regulate expression in leukemic cells. Our data shows chemical inhibition or genetic knockdown of Prmt5 extends AML *in vivo* consistent with recent work implicating *Prmt5* in the progression of hematologic malignancies[31-34]. Our data identify the PAFc as a direct regulator of the *Prmt5* locus, connecting the PAFc to the deposition of H4R3me2s. Together, these data place the PAFc atop a pro-leukemogenic gene program that includes, not only *Hoxa9* and *Meis1*, but also *Prmt5* and illustrates the potential of therapeutic targeting the PAFc and Prmt5 in AML.

Results

Conditional Excision of *Cdc73* Induces Differentiation and Alters the Histone Modifications of AML Cells

To evaluate the role of the PAFc in leukemic cells, we utilized a *Cdc73* floxed mouse to generate MLL-AF9 AML cell lines containing a tamoxifen (4OHT) inducible CreER (MA9-*Cdc73fl*-CreER) and control cell lines (MA9-CreER) (Figure 1A, S1A). Our past work demonstrates that conditional excision of the PAFc subunit *Cdc73*, reduces the proliferation of MA9 leukemic cells and MLL fusion protein target gene expression[3]. We sought to explore the cellular phenotypes associated with *Cdc73* excision that would elucidate its role in leukemia. Upon 4OHT treatment, MA9-*Cdc73fl*-CreER cells displayed morphology changes consistent with cellular myeloid differentiation including increased cytoplasmic to nuclear ratios (Figure 1B) and reduced c-Kit and increased Cd14 cell surface expression (Figure 1C). qPCR analysis of genes associated with myeloid differentiation (*Mmp8*, *Mmp12*, *Id2*, *Ltf* and *Cd80)* showed upregulation upon *Cdc73* excision (Figure 1D)[35, 36]. Cell cycle analysis revealed *Cdc73* excision results in a G1 phase cell cycle block (Figure 1E), while changes in apoptosis were mild (Figure S1B). We analyzed global histone modifications in MA9-*Cdc73fl*-CreER cells and detected a reduction of H3K4me3, H3K79me2, and an almost complete ablation of H2BK120ub following excision of *Cdc73* (Figure 1F). Additionally, loss of *Cdc73* reduced colony formation of MA9-*Cdc73fl*-CreER cells (Figure S1C). Overall, these data suggest that loss of Cdc73 partially induces differentiation of leukemic cells and alters global H3K4me3, H3K79me2 and H2B120ub.

Prmt5 Expression is Dependent on *Cdc73* in MLL-AF9 Cells

Next, we asked what gene programs were controlled by the PAFc that may contribute to blocking differentiation of leukemic cells. To that end, we performed RNA-sequencing using our MA9-*Cdc73fl*-CreER and MA9-CreER cell lines. Cell lines were treated with 4OHT for 48 hours and RNA harvested for sequencing (Figure 2A). To control for gene expression differences between cell lines or variation in MLL-AF9 expression, we also isolated RNA from the MA9-*Cdc73fl*-CreER cell line treated with EtOH (Figure 2A). A comparison of gene expression changes in MA9-*Cdc73fl*-CreER cells treated with EtOH or 4OHT (EtOH control) to MA9-*Cdc73fl*-CreER and MA9-CreER cells treated with 4OHT (Cre control) revealed a slope of 0.98 (Figure 2B) revealing remarkably similar gene expression changes. This indicates the changes in gene expression are in response to loss of *Cdc73*. We observed that only a subset of genes were significantly changed (fold-change > 1.5, FDR q-value <0.01) and that genes were both upregulated and downregulated upon *Cdc73* excision

perhaps pointing to a role for the PAFc in both activation and repression (Figure 2C)[37]. We report 1835 upregulated and 2151 downregulated genes at 48 hours following *Cdc73* excision.

To determine whether the genes altered by loss of *Cdc73* are associated with specific gene programs or pathways, we performed GAGE analysis. Upon *Cdc73* excision, several gene pathways associated with differentiation such as immune response were enriched in our upregulated gene set, while methyltransferase activity and transfer of methyl groups were enriched in the downregulated gene set (Figure S2A). To explore specific gene programs regulated by the PAFc in leukemic cells we also utilized Gene Set Enrichment Analysis (GSEA). Consistent with the differentiation phenotype described above, excision of *Cdc73* increased expression of a gene program associated with myeloid development while control MA9-CreER cells expressed an early hematopoietic progenitor program along with a Hoxa9/Meis1 gene program characteristic of MLL-fusion driven leukemias (Figure 2D). Interestingly, 25 of 129 direct MLL-AF9 target genes and 42 of 165 direct MLL-ENL targets showed significant expression changes upon loss of *Cdc73* in our MA9-*Cdc73fl*-CreER cells [38, 39] (Table S3, S4, S5). These data are suggestive of PAFc functions that are both cooperative and independent of MLL fusion proteins. Consistent with the GAGE analysis, expression of a methyltransferase activity program was enriched in genes that were downregulated in MA9-*Cdc73fl*-CreER cells following 4OHT treatment (Figure 2D). Among the genes altered in the methyltransferase activity program were several members of the protein arginine methyltransferase (*Prmt*) family. Thus, we investigated the fold change of *Prmt* genes with an RPKM > 5 following excision of *Cdc73* and observed most were downregulated (Figure S2B).

We next compared our mouse *Cdc73* excision expression data set with a knockdown dataset from human leukemic THP-1 cells targeting another PAFc subunit, *PAF1* and discovered both unique and overlapping genes from the up and downregulated groups (Figure 2E, Table S2)[40]. Among the commonly downregulated genes *PRMT5, PRMT1*, and *PRMT3* were all significantly downregulated following loss of either *Cdc73* or *PAF1* ($q<0.05$) (Figure 2F). These data implied that not only are these genes most likely regulated by the PAFc vs a CDC73 specific target, but the regulation is conserved between mouse and human. Next, we compared how *Prmt* expression differs between normal and leukemic cells. While most *Prmt* genes are upregulated in MLL-AF9 cells compared to normal $lin^{-}c\text{-}kit^{+}$ mouse bone marrow cells, *Prmt5* showed one of the highest percentage increases in expression (Figure 2G). Taken together, our analysis directed us to investigate the role of several Prmt's in MLL-fusion leukemia.

Prmt5 is Necessary for Leukemia Cell Growth *In Vitro* and *In Vivo*

To investigate the role of Prmt genes in leukemia, we narrowed our evaluation to *Prmt1*, *Prmt4*, and *Prmt5* based on several criteria: 1) the identification of *Prmt1 and Prmt5* in both *PAF1* and *Cdc73* expression analyses (Figure 2F), 2) genetic deletion of *Prmt1, Prmt4*, and *Prmt5* shows embryonic lethality in mice suggesting non-overlapping function and 3) reported roles of all three in various cancers including leukemia[31-34, 41-43]. We confirmed our RNA-seq with qPCR, which showed downregulation of *Prmt1, Prmt4*, and *Prmt5*

following loss of *Cdc73* (Figure S2C). We generated constitutively active pSM2C-shRNA vectors targeting these *Prmts* and validated them in MA9 cells by qPCR (Figure S3B, top panel). Knockdown of *Prmt1, Prmt4*, and *Prmt5* results in a significant decrease in cell number of MLL-AF9 transformed cells (Figure S3C). However, a significant decrease of colony formation was only observed following knockdown of *Prmt4* and *Prmt5* (Figure S3D). Interestingly, only overexpression of *Prmt5* significantly increased proliferation and colony formation in MA9 cells (Figure S3E, S3F)[44]. Since knockdown and overexpression of *Prmt5* showed significant phenotypic effects and we identified this gene in both *PAF1* and *Cdc73* expression data sets, we focused on the role of Prmt5 in leukemic cells and its regulation by the PAFc.

We generated an inducible TetON *Prmt5* knockdown system to more precisely assess the function of Prmt5 in leukemic cells. We observed reduced mRNA and protein following shRNA induction (Figure S3A, S3B lower panel). We performed competition assays using MA9-TetON-sh*Prmt5* or control MA9-TetON-sh*Renilla* cell lines cocultured with parental MA9-TetOn cells. Cells with *Prmt5* knockdown were outcompeted by parental MA9-TetON cells while those expressing the sh*Renilla* construct remained stable through the experiment (Figure 3A-B). This illustrates a competitive growth disadvantage in leukemic cells with reduced *Prmt5* expression. Similarly, growth assays show knockdown of *Prmt5* causes a proliferative defect in MA9 cells (Figure 3C). Interestingly, c-Kit and Cd14 cell surface expression only mildly change after *Prmt5* knockdown suggesting the differentiation of leukemic cells observed following excision of *Cdc73*, (Figure 1) is not facilitated by *Prmt5* (Figure 3D). However, reduction of *Prmt5* does lead to G1 cell cycle arrest similarly to *Cdc73* excision suggesting *Prmt5* contributes to cell cycle progression in leukemic cells downstream of the PAFc (Figure 3E).

We next used our inducible knockdown system to test how loss of *Prmt5* affects AML disease latency in vivo. To examine the role of *Prmt5* in a broader group of AML, we knocked down expression in leukemic cells driven by overexpression of Hoxa9 and Meis1, which are direct MLL-fusion targets overexpressed in ~50% of AML[24]. Here, we transduced primary TetON Hoxa9/Meis1 leukemic cells with TRMPV-sh*Prmt5* vectors and injected them into syngeneic recipient mice followed by knock down of *Prmt5* with doxycycline (Dox). We verified that cells showed activated shRNA and knocked down *Prmt5* prior to injection (Figure S4A). Dox mediated *Prmt5* knockdown significantly extended disease latency by 4.5 days compared to our non-Dox treated group (Figure 3F). Leukemic cell infiltration in the liver, spleen, and bone marrow was comparable between both groups as was the spleen weight at the moribund state (Figure S4B-D). Interestingly, we observe similar levels of *Prmt5* expression suggesting a selective pressure to maintain *Prmt5* expression (Figure S4E). We repeated this assay using MA9-TetON cells and see no statistical latency extension between our treatment groups (Figure S5A, B). We observe a significant reduction in spleen weight in the Dox treated group at the moribund state, but comparable levels of leukemic cells in the BM, tissue infiltration, and *Prmt5* expression (Figure S5C-F). The long disease latency in the MLL-AF9 model likely provides additional time to select cells that escape *Prmt5* knock down. These data indicate an important role for Prmt5 in promoting cell cycle progression and leukemogenesis in AML displaying overexpression of the Hoxa9/Meis1 gene program such as MLL fusion leukemias.

MLL-AF9 Cells are Dependent on PRMT5 Enzymatic Activity

We next asked whether the enzymatic activity of Prmt5 was required. We transduced our MA9-TetOn-sh*Prmt5* cells with either wild-type PRMT5 (WT-P5) or a catalytic dead PRMT5 mutant (CD-P5) as described[27], and subjected them to growth assays. While the growth defect in MA9-TetON-sh*Prmt5* cells after *Prmt5* knockdown is rescued by WT-P5 expression, the CD-P5 mutant did not suggesting the enzymatic activity of Prmt5 is necessary for MLL-AF9 leukemic cell growth (Figure 4A). Thus, we investigated histone modifications in MLL-AF9 cells following knockdown of *Prmt5*. Western blot analysis revealed global reduction of pan symmetric di-methylation of arginine (SDMA) and H4R3me2s following knockdown of *Prmt5* with little change on H3K4me3, H3K79me2, and H2Bub (Figure 4B). We similarly detected reduced H4R3me2s and pan SDMA after *Cdc73* excision consistent with the PAFc regulating Prmt5 (Figure 4C). This effect was specific since H4R3 asymmetric methylation (H4R3me2a) catalyzed by Prmt1 and Prmt6 remained unchanged (Figure S6B). Additionally, knockdown of the PAF1 subunit in HeLa cells also reduces PRMT5 protein level consistent with the PAFc regulating *PRMT5* expression (Figure S6D). To test whether *Prmt5* overexpression can rescue proliferation following loss of *Cdc73*, we stably transduced WT-Prmt5 and CD-Prmt5 into our MA9-*Cdc73fl*-CreER cells. Ectopic expression of neither WT-Prmt5 nor CD-Prmt5 rescues the growth of cells lacking *Cdc73* consistent with a multitude of important PAFc target genes (Figure S6A). Because Prmt5 knockdown did not completely arrest MA9 cell growth, we asked whether this might be due to escapees. To this end, we maintained MA9-TetON-sh*Prmt5* cells in Dox for 9 days and monitored Prmt5 expression by western blot. Prmt5 protein expression does recover after initial knockdown suggesting a selective pressure for maintaining Prmt5 expression in leukemic cells (Figure 4D and S6C). These data imply that MLL-AF9 leukemic cells require the PRMT5 protein, and its enzymatic activity to maintain and promote oncogenesis.

The PAFc is a Direct Transcriptional Regulator of the *Prmt5* Locus

In yeast, the PAFc regulates about 20% of genes[45]. Similarly, in human cells PAF1 knockdown only modestly changed gene expression suggesting the PAFc governs a specific program[40]. To investigate whether the PAFc directly regulates *Prmt5*, we analyzed ChIP-sequencing data targeting the PAFc generated from the human MLL-AF9 driven AML cell line, THP-1[40]. We observed a distinct binding peak of the PAFc, represented by CDC73 and LEO1 that overlaps with RNA polymerase II at the transcriptional start site of the *PRMT5* gene (Figure 5A). To confirm this enrichment in mouse cells, we performed ChIP-qPCR experiments on the *Prmt5* locus in MA9-*Cdc73fl*-CreER leukemic cells with and without *Cdc73* excision (Figure 5B). Cdc73, Leo1, Mll1c, and MLL-AF9 were enriched at the *Prmt5* locus. *Cdc73* excision reduces enrichment of all four proteins suggesting Cdc73 is necessary for the localization of these proteins to *Prmt5* (Figure 5C), similar to the known MLL and PAFc target gene *Meis1* (Figure S7A and S7B). Loss of *Cdc73* also leads to a decrease in H3K4me3 consistent with reduced transcriptional activity (Figure 5C). Consistent with the PAFc regulating *Prmt5* expression, a *Prmt5*-Luciferase reporter was activated in a dose dependent manner to increasing levels of PAFc in the presence of MLL-AF9 (Figure S7C). Recent work has shown STAT5 is a direct regulator of *PRMT5*[32]and overlaps with HOXA9 binding sites[46]. Thus, we analyzed HOXA9 and STAT5 binding at the *Prmt5* locus. To test

this, we generated 4OHT inducible *Cdc73 floxed* leukemic cell lines transformed with Myc-HOXA9 and HA-MEIS1. We observed Stat5 binding at the *Prmt5* transcriptional start site that was strongly dependent on Cdc73 (Figure 5D). Ectopically expressed Myc-HOXA9 also enriches at the *Prmt5* locus, but was less responsive to *Cdc73* excision (Figure 5D). These data support that *Prmt5* is directly regulated by the PAFc, which promotes expression in conjunction with other oncogenic factors such as STAT5 and HOXA9 in leukemic cells.

Chemical inhibition of Prmt5 Reduces Leukemic Cell Growth and Prolongs Disease Latency In Vivo

PRMT5 inhibitors are currently in clinical trials for the treatment of solid tumors (ClinicalTrials.gov Identifier: NCT02783300). To understand how chemical inhibition of Prmt5 activity affects leukemic cell growth *in vitro* and *in vivo*, we treated several human leukemic cell lines with the PRMT5 inhibitor EPZ015666 and measured proliferation[31]. U937, NB4, and MonoMac6 cells all display a dose dependent decrease in proliferation and H4R3me2s demonstrating multiple leukemic subtypes are affected by PRMT5 inhibition (Figure 6A). Notably, NB4 cells, which display low MTAP expression, also show the greatest sensitivity to PRMT5 inhibition (Figure S8A). This is remarkably consistent with recent reports revealing cancer cell dependence on PRMT5 with the loss of the MTAP enzyme [47, 48]. Similarly, mouse MLL-AF9 leukemic cells showed a dose dependent decrease in proliferation and H4R3me2s following Prmt5 inhibition (Figure S8B-C). We investigated how AML responds to Prmt5 inhibition using the EPZ015666 compound in an MLL-AF9 mouse model. Primary MLL-AF9 leukemic cells were injected into syngeneic recipients and dosed with EPZ015666 for 14 days. The median survival of mice receiving MLL-AF9 cells was extended by 4.5 days with EPZ015666 treatment (Figure 6B). While all moribund mice succumbed to leukemia (Figure 6B), reduced spleen weight and leukemic infiltration in EPZ015666 treated mice points to a less progressed disease compared to vehicle treated mice (Figure 6C and S9A-B). Potentially, these mice are more susceptible to leukemic burden due to drug toxicity, consistent with control mice tolerating the dosing schedule, but exhibiting minor weight loss (Figure S9C). We see no difference in H4R3me2s methylation at the moribund state between both treatment groups that likely reflects a strong selective pressure (Figure S9D). These results imply Prmt5 may play an important role in multiple subtypes of AML where chemical inhibition of PRMT5 may be an effective treatment.

Discussion

We examined the downstream gene program controlled by Cdc73 in leukemic cells and discovered that *Prmt5* is a direct transcriptional target of the PAFc. Similar to yeast studies, we observed that conditional excision of *Cdc73*, as a surrogate for the PAFc, transcriptionally altered only a subset of genes suggesting specific gene programs under control of the PAFc[45]. Indeed, our data points to the PAFc maintaining gene programs that promote self-renewal and proliferation (Figure 2). Many genes from our data set were also upregulated, potentially reflecting indirect transcriptional changes associated with differentiation following loss of *Cdc73* or a role for the PAFc in repressing transcription as has been reported[37]. Importantly, we can assign several *Prmt* genes to the list of PAFc targets. Past studies describe the importance of the PAFc or its individual components, as

well as PRMT proteins in a variety of cancers including MLL-rearranged leukemias[2, 4, 44, 49]. Thus, a thorough understanding of the transcriptional mechanisms regulating *PRMT* genes may be used to develop novel therapies.

PRMT5 is a protein arginine methyltranferase necessary to deactivate gene transcription of targets and has a growing list of non-histone target proteins[25, 27, 50-52]. Studies exploring the overexpression of *PRMT5* in cancer have clearly established a pro-oncogenic role which may be relevant in a diverse list of cancer types[28, 33, 53, 54]. Our work describes the transcriptional regulation of *Prmt5* by the PAFc (Figure 5). The identification of selective cellular phenotypes associated with loss of *Prmt5* (Figure 3), suggests several functional pathways lie downstream of the PAFc in leukemia. For example, while differentiation and cell cycle arrest were prevalent upon *Cdc73* excision, only cell cycle arrest occurred with direct *Prmt5* knockdown (Figure 1 and 3). This suggests that upregulation of known PAFc targets *Hoxa9* and *Meis1*[3, 4] are predominantly responsible for blocking myeloid differentiation, while PAFc mediated regulation of *Prmt5* contributes to cell cycle progression. In addition to the PAFc, we detected MLL-AF9, MLL1, STAT5, and HOXA9 at the *Prmt5* locus (Figure 5). Loss of *Cdc73* does not affect ectopically expressed HOXA9 enrichment at the *Prmt5* locus. Thus, Hoxa9 may be a pioneering transcription factor that associates with targets independent of the PAFc. Additionally, these data are consistent with STAT protein dependence on CTR9 for localization to IL-6 responsive genes[55].

It is noteworthy that several *Prmt* genes were deregulated after *Cdc73* excision, although only *Prmt5, Prmt1*, and *Prmt3* were commonly misregulated in mouse and human cells following disruption of the PAFc (Figure 3). Thus, the PAFc may help regulate multiple *PRMT* genes with oncogenic functions, which will require further investigation. Still, our data and others point to PRMT5 (amongst several PRMT proteins) as being critically important to facilitate leukemia[56]. For example, PRMT1, PRMT4, and PRMT5 have all been reported to promote leukemia through a variety of mechanisms[31-34, 41-43, 57, 58,59]. However, some of these are also essential during development[56]. For example, loss of *Prmt5* is embryonic lethal and conditional knockouts demonstrate it is essential for the maintenance of adult hematopoietic stem cells[30, 51]. Further, loss of *Prmt1* or *Prmt4* is lethal during embryogenesis or shortly after birth[60, 61]. Thus, targeting the transcriptional and epigenetic mechanisms regulating several *PRMT* genes, such as the PAFc, may allow for a combinatorial downregulation of *Prmt* genes without fully abolishing expression, which may be therapeutically advantageous. This is highlighted by our results that chemical inhibition of PRMT5 extends disease latency, but does not cure the leukemic disease (Figure 6). A combinatorial approach targeting the PAFc may facilitate altered expression of target *PRMT* genes that avoids tissue toxicity while exploiting the reliance of tumor cells on high *PRMT* expression. Further, targeting of the PAFc would also downregulate *Hoxa9* and *Meis1* that serve as oncogenic drivers in as much as 50% of AML[24]. Our previous work established the PAFc-MLL interaction as an attractive therapeutic target. Our detection of MLL and MLL-fusion proteins at the *Prmt5* locus suggests that targeting of the PAFc-MLL interaction may alter *Prmt5* expression in addition to *Hoxa9* and *Meis1*. Overall, the work presented here suggests *Prmt5* is one of many potential PAFc targets contributing to the progression and maintenance of MLL driven leukemia and supports the therapeutic potential of the PAFc in leukemias and other cancer types.

Materials and Methods

Mice

Female C57BL/6 mice at 8-10 weeks old were purchased from Taconic Farms (Hudson, NY). B6.Cg-Gt(ROSA)26Sortm1(rtTA*M2)Jae/J mice (TetOn mice) were purchased from Jackson laboratory (Bar Harbor, ME). All animal studies were approved by the University of Michigan Committee on Use and Care of Animals and the Unit for Laboratory Medicine.

Colony assays, cell lines and PRMT5 inhibition assay

Colony assays and cell line generation were performed as previously reported[3, 62]. MA9-*Cdc73fl*-CreER and MA9-CreER cells were treated with 7.5nM 4OHT for 48 or 72 hours to induce excision of *Cdc73*. For PRMT5 chemical inhibition, 10K cells were subjected to various doses of EPZ015666, counted every 4 days (3 days for MA9 cells), retreated and reseeded at original density for a total of 12 days as described [31]. For shRNA knockdown, MA9 cells were re-transduced with pSM2C-sh*Prmt5* or control vectors and selected in puromycin (100ng/mL), plated and counted after 1 week. Prmt overexpression assay cells were re-plated for 3 rounds. Transforming TetOn mouse bone marrow with the MLL-AF9 oncogene generated stable TetON cell lines as described [62]. Knockdown was achieved by transducing with the pTRMPV vector containing shRNA directed towards *Prmt1*, *Prmt4*, *Prmt5* and *Renilla* control (Table S1) and Doxycycline treatment (1μg/ml). shRNA constructs were designed as described[63]. Differentiation, cell cycle, and apoptosis assays were performed as described [62] using c-Kit, Cd14, Cd11b and isotype control antibodies (BD Pharmigen, San Jose, CA & Biolegend, San Diego, CA) or Annexin V-DAPI (BD Pharmigen, San Jose, CA) co-stain and read on an LSRII Flow Cytometer (BD Biosciences, San Jose, CA). Data was analyzed using FlowJo X and ModFit Software. siRNA knockdown of Paf1 in MA9-Cdc73fl-CreER cells was performed as previously described[4].

RNA Sequencing and ChIP sequencing

MA9-*Cdc73*fl-CreER and MA9-CreER cells were treated with 7.5nM 4OHT and harvested at 48 hours. RNA-seq libraries were prepared according to the manufacturer's instructions (Illumina, Eindhoven, The Netherlands) by the University of Michigan Sequencing Core. All data available at the Gene Expression Omnibus: GSE90136. Previously published RNA-seq and ChIP-Seq reads were downloaded from project number SRP048744 in GEO and converted to fastq format using the SRA toolkit[40].

In Vivo Disease Latency

Primary Hoxa9/Meis1 transformed TetOn leukemic cells were transduced with either TRMPV-shRenilla or sh*Prmt5* packaged retrovirus, selected in 200 μg/mL hygromycin and injected (250K cells) into irradiated (600 rads) syngeneic recipients. Dox food (Harlan Labs 625mg/kg) and water (Sigma 2mg/mL) were used to induce knockdown. For Prmt5 chemical inhibition *in vivo*, mice received EPZ015666 by oral gavage at 200 mg/kg BID for 14 days[31], 4 days post MA9 cell injection. Mice were sacrificed and analyzed at the moribund stage as described [62]. Mice were block randomized at N=7 after power analysis

and investigator blinding was not used. No mice were excluded due to failure of cell engraftment following irradiation.

ChIP-qPCR

ChIP-qPCR was performed as described[64], using αCDC73, αLEO1 (Bethyl, Montgomery, TX), αMLLc (gift from Dr. Yali Dou), αFlag (Sigma), αH3K4me3, αH3 (Abcam, Cambridge, UK), IgG (Santa Cruz, Dallas, TX), Stat5-Y694 and Myc-Tag-71D10 (Cell Signaling, Danvers, MA). qPCR primer sequences are listed (Table S1).

Western Blots

Western blots were performed with whole cell lysates or acid extracted histones and probed with the following antibodies: αCDC73, αPAF1 (Bethyl, Montgomery, TX), αPRMT5, αSYM10, αGAPDH, αH2BUb (Millipore, Billerica, MA), αH3, αH3K4me3, αH3K79me2, αH4R3me2s (Abcam,Cambridge, UK), αR-Me2s (Cell Signaling, Danvers, MA), α-βActin (Sigma). Bands were detected using SuperSignal West Pico Chemiluminescent substrate (Thermo-Fisher Scientific, Waltham, MA) and imaged on a ChemiDocXRS (Bio-Rad, Hercules, CA).

Statistical Validation & Human Cell Lines

Statistics, sample sizes, replicates, and p-values are presented in figure legends or main text. Variance was factored into statistical tests where applicable. U937 sourced from ATCC while NB4 and MM6 were sourced from DSMZ. All were STR validated, but not tested for mycoplasma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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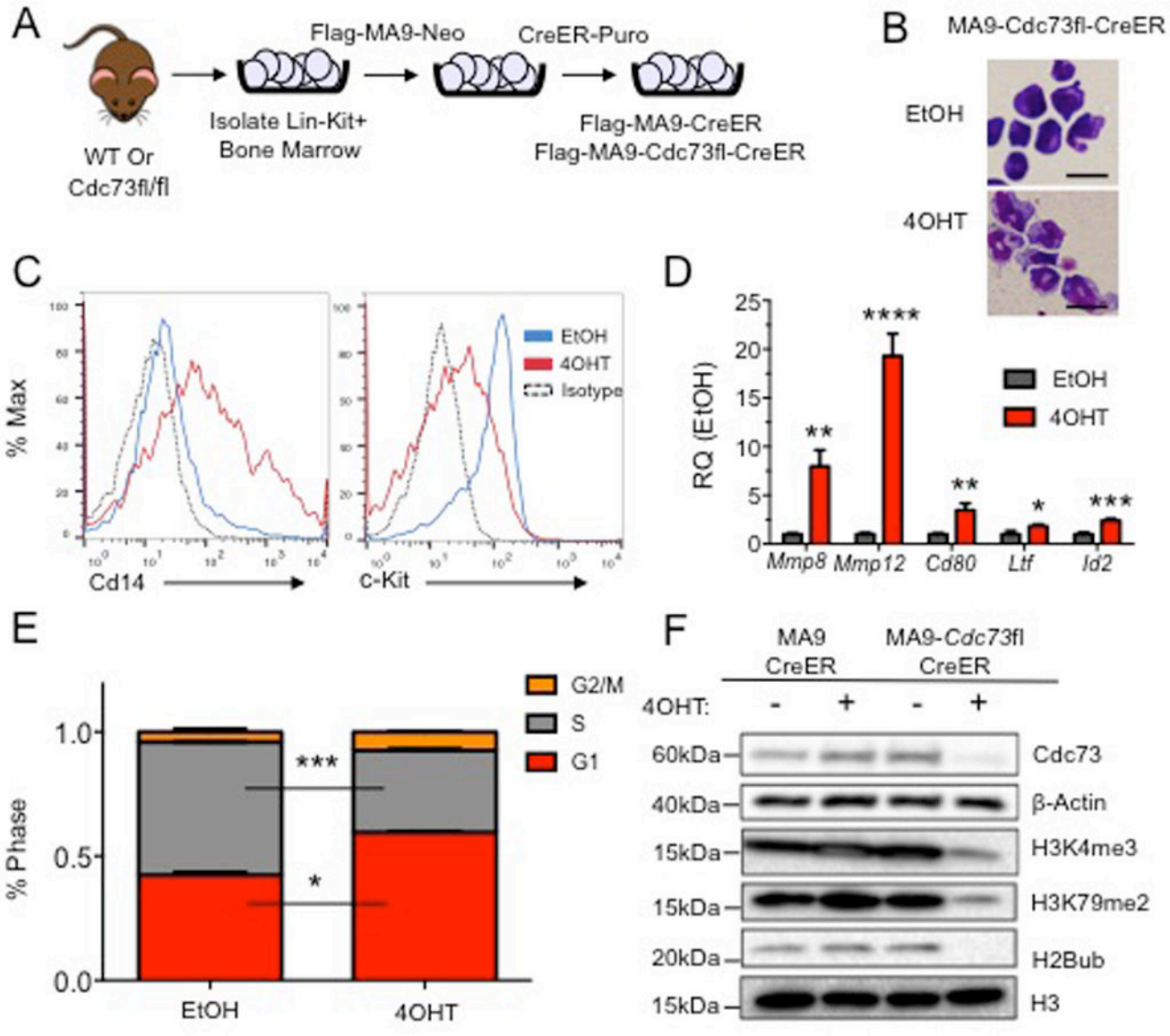
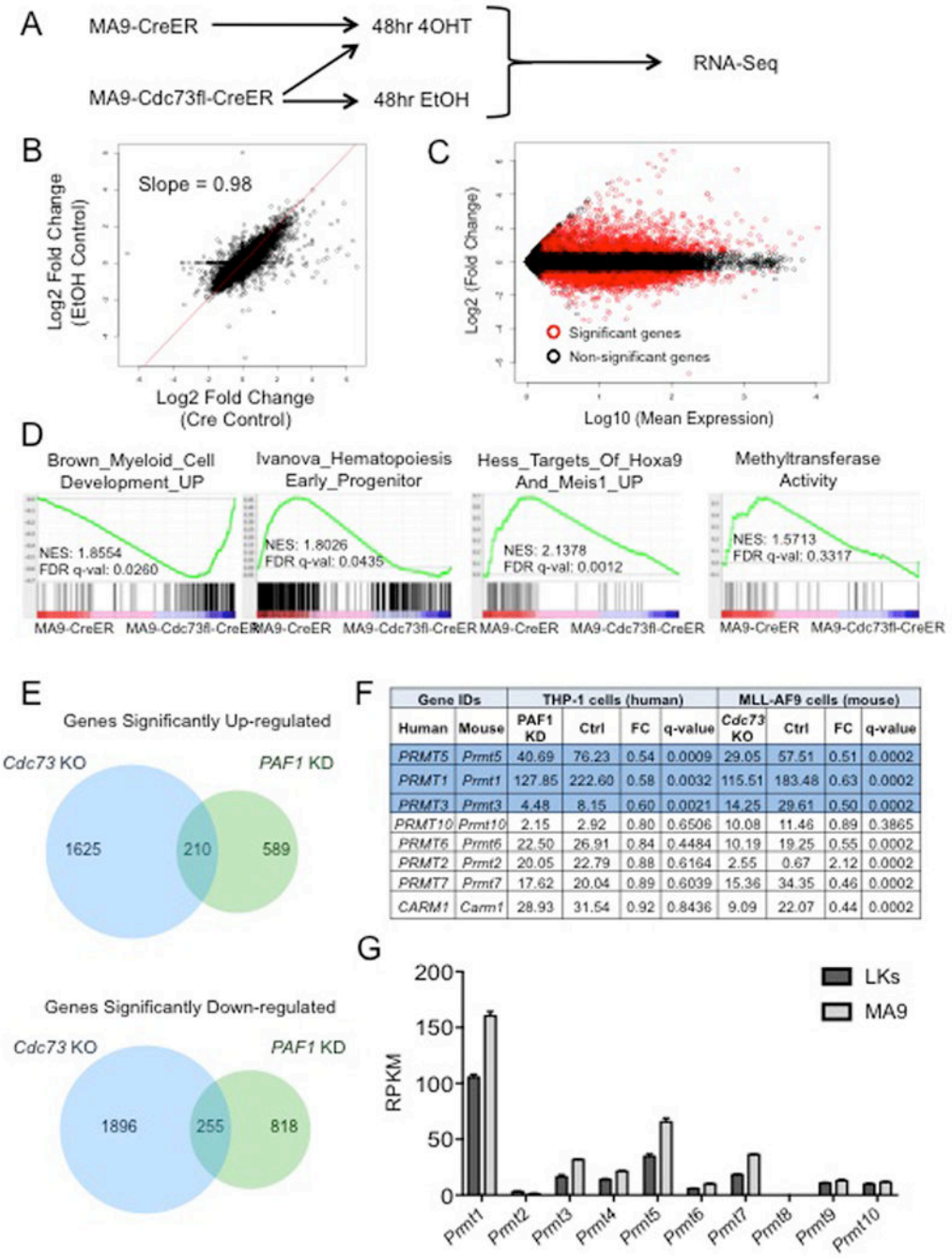


Figure 1. Conditional Excision of *Cdc73* Induces Differentiation and Alters Epigenetic Landscape in MA9 Leukemic Cells

A) Scheme showing generation of Flag-tagged MA9 leukemic *Cdc73* excision cells driven by inducible CreER for phenotypic analysis. B) MA9-*Cdc73*fl-CreER cells were treated with either EtOH or 7.5nM 4OHT for 48 hours, cytospun onto slides and stained with the Hema3 staining kit. Representative images at 100× show morphology changes. Scale bar = 20μM C) Excision of *Cdc73* increases level of differentiation as measured by c-Kit, Cd14 cell surface markers on MA9 leukemic cells by flow cytometry. D) *Cdc73* excision increases differentiation gene signature expression including *Mmp8*, *Mmp12*, *Cd80*, *Ltf* and *Id2*. All samples were scaled to β-actin and normalized to EtOH control expression. * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001, **** = p-value < 0.0001. Student t-test. E) *Cdc73* excision results in a G1 cell cycle block. MA9 cells were treated as in (B) and cell cycle analysis performed using ModFit software. * = p-value < 0.05, *** = p-value < 0.001

Student t-test. F) Excision of *Cdc73* alters global epigenetic landscape. Cells were treated as in (B), and ~2×10^6 cells were harvested after 72 hours and subjected to histone extraction and probed with the indicated antibodies. H3 and β-Actin are used as loading controls. All experiments were performed in biological duplicate and technical duplicate for phenotypic analysis or technical triplicate for qPCR.



Gene IDs		THP-1 cells (human)				MLL-AF9 cells (mouse)			
Human	Mouse	PAF1 KD	Ctrl	FC	q-value	Cdc73 KO	Ctrl	FC	q-value
PRMT5	*Prmt5*	40.69	76.23	0.54	0.0009	29.05	57.51	0.51	0.0002
PRMT1	*Prmt1*	127.85	222.60	0.58	0.0032	115.51	183.48	0.63	0.0002
PRMT3	*Prmt3*	4.48	8.15	0.60	0.0021	14.25	29.61	0.50	0.0002
PRMT10	*Prmt10*	2.15	2.92	0.80	0.6506	10.08	11.46	0.89	0.3865
PRMT6	*Prmt6*	22.50	26.91	0.84	0.4484	10.19	19.25	0.55	0.0002
PRMT2	*Prmt2*	20.05	22.79	0.88	0.6164	2.55	0.67	2.12	0.0002
PRMT7	*Prmt7*	17.62	20.04	0.89	0.6039	15.36	34.35	0.46	0.0002
CARM1	*Carm1*	28.93	31.54	0.92	0.8436	9.09	22.07	0.44	0.0002

Figure 2. Prmt5 Identified as a Potential Downstream Target Gene of the PAFc

A) Scheme used for RNA-sequencing. B) Scatter-plot of changed genes between MA9-*Cdc73*fl-CreER 4OHT treated cells compared to either EtOH or CreER controls. Best fit displayed with slope. C) MA plot at 48 hours post 4OHT treatment showing fold change of genes in excision cells over MA-CreER control relative to the average RPKM value of the given gene across all samples. Red circles represent genes with 1.5 fold change up or down and a q-value < 0.01. D) GSEA analysis shows MA9 samples aligns with early progenitor and Hoxa9/Meis1 targets as expected while *Cdc73* excision cells align with a myeloid

differentiation gene set. Genes expressed in MA9 cells enrich with a methyltransferase gene program that is lost after excision. NES and q-value are displayed. E) Venn diagrams depict both overlapping and distinct genes are up and down regulated with excision of *Cdc73* and knockdown of *PAF1*. Comparison of our *Cdc73* excision dataset with a *PAF1* KD data set in human THP-1 (MLL-AF9 driven) leukemic cells displayed 210 overlapping upregulated genes and 255 overlapping down-regulated genes. F) Table shows Prmt family gene expression changes following knockdown of *PAF1* in THP1 cells and excision of *Cdc73* in MA9 cells. Blue rows indicate genes, which displayed significant expression changes (FC > 1.5, q-value < 0.01) in both Cdc73 and PAF1 datasets. G) The Prmt family is over-expressed in MA9 leukemic cells relative to normal $Lin^{-}Kit^{+}$ bone marrow by RPKM using an FDR < 0.5. RNA-sequencing samples were submitted in biological duplicate.

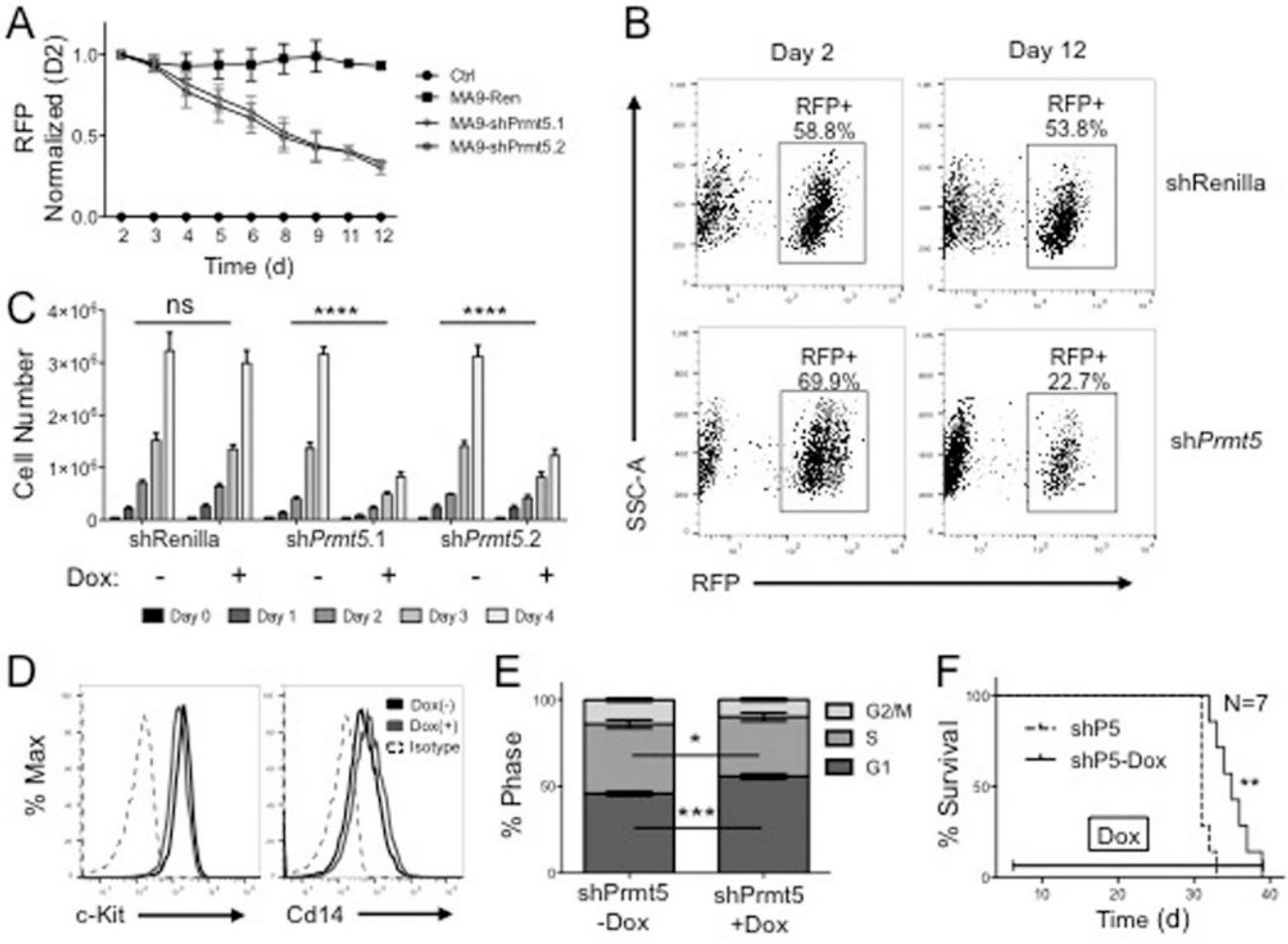


Figure 3. Modulation of *Prmt5* Disrupts Leukemic Progression in MA9 Cells

A) Loss of *Prmt5* reduces cell fitness. Cells were plated in duplicate in a 3:1 ratio of parent MA9-TetON cells to MA9-TetON sh*Prmt5* knockdown cells and treated with vehicle or Dox. RFP signal was read every two days by flow cytometry. B) Gating of RFP readout is shown at Day 2 and Day 12. C) *Prmt5* knockdown reduces cell proliferation. Cells were treated with 1μg/mL Dox once per day and grown for 4 days. shRenilla is used as a control. **** = p-value < 0.0001 by Two-way ANOVA. D) *Prmt5* knockdown has minimal effect on differentiation. Cells were treated as in (C) and harvested at 48 hours, incubated with c-Kit or Cd14 antibody and analyzed by flow cytometry. E) Loss of Prmt5 leads to G1 cell cycle arrest. Cells were treated as in (C) and stained with DAPI and analyzed by flow cytometry. * = p-value < 0.05, *** = p-value < 0.001 by two-tailed student's t-test. F) Prmt5 knockdown extends disease latency in Hoxa9/Meis1 leukemic model. Survival curve of a secondary transplant of TetON-MA9-sh*Prmt5* cells transplanted into sub-lethally irradiated recipients treated with vehicle or Dox beginning on day 4. N=7, ** = p-value < 0.01 by Wilcoxon test. All experiments were performed in biological duplicate and technical duplicate.

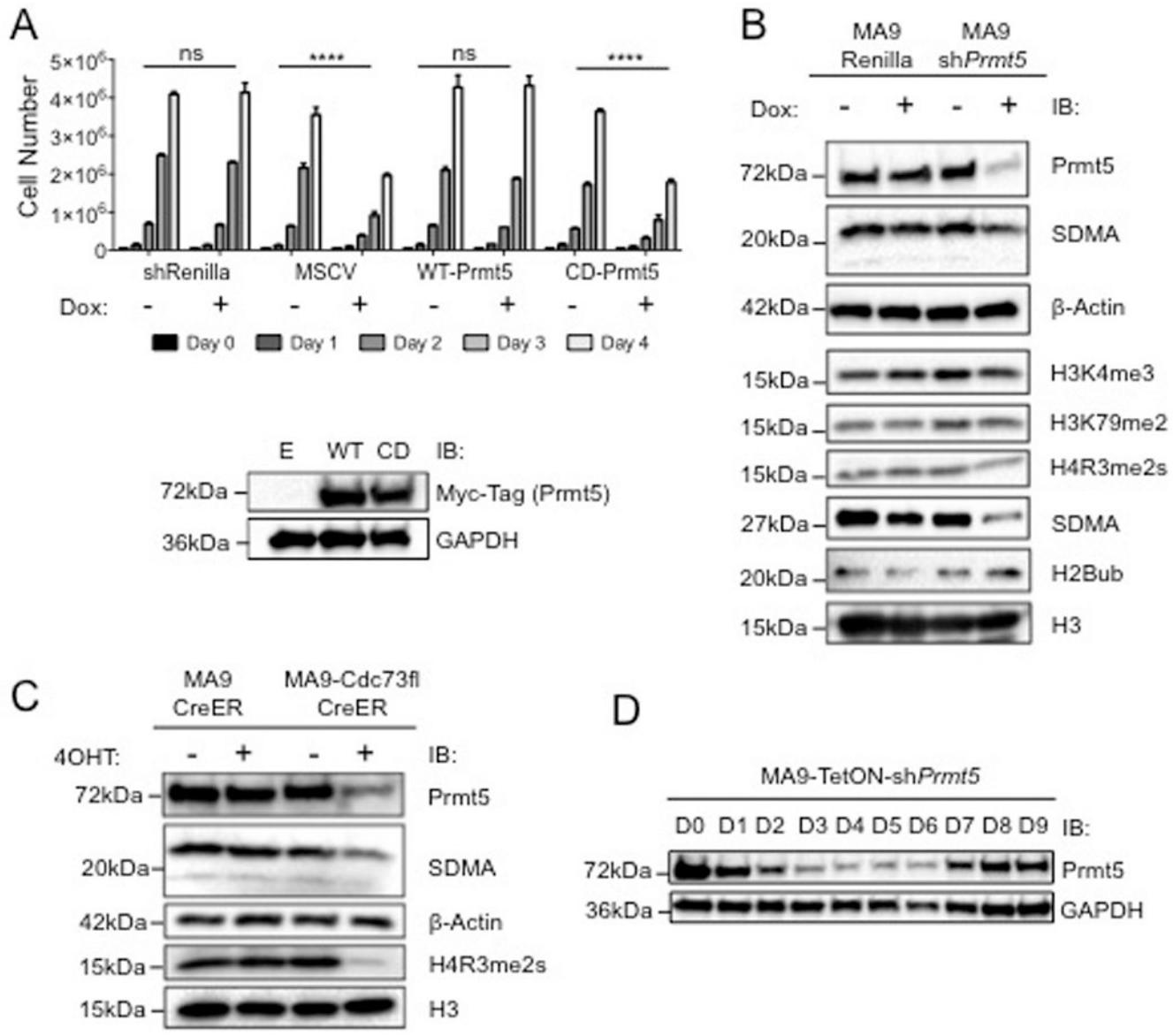


Figure 4. The catalytic activity of Prmt5 is Required for MA9 leukemic cells

A) Expression of catalytic dead Prmt5 mutant cannot rescue growth of MA9 cells. WT-Prmt5 or CD-Prmt5 were transduced into MA9-TetON-shPrmt5 cells to generate stable lines. Cells were treated with either PBS or Dox every day for 4 days. **** = p-value < 0.0001 by Two-way ANOVA. Performed in biological triplicate and technical triplicate. Protein level of expression vectors is shown below. B) Knockdown of *Prmt5* specifically alters its histone modification targets. MA9-TetON cells containing either sh*Renilla* or sh*Prmt5* were treated with 1μg/mL Doxycycline every day for 3 days followed by histone extraction or whole cell extraction and western blots. Data indicates global reduction of SDMA and H4R3me2s without changes to H3K4me3, H3K79me2 and H2Bub. Prmt5 antibodies were used as confirmation of knockdown with whole cell lysate. H3 and β-Actin are used as loading controls. C) *Cdc73* excision in MA9 cells reduces PRMT5 histone

modification targets. Cells were treated as in (B) with the exception of 7.5nM EtOH or 4OHT instead of doxycycline. Reduced H4R3me2s and SDMA is observed. H3 and β-Actin are used as loading controls. D) MA9 cells show strong selective pressure to maintain PRMT5. Cells were maintained in the presence of 1μg/mL Dox and passaged as needed. 1×10^6 cells were harvested per day and PRMT5 level in the lysate was measured with Prmt5 antibody and GAPDH as a loading control. Westerns performed in biological duplicate.

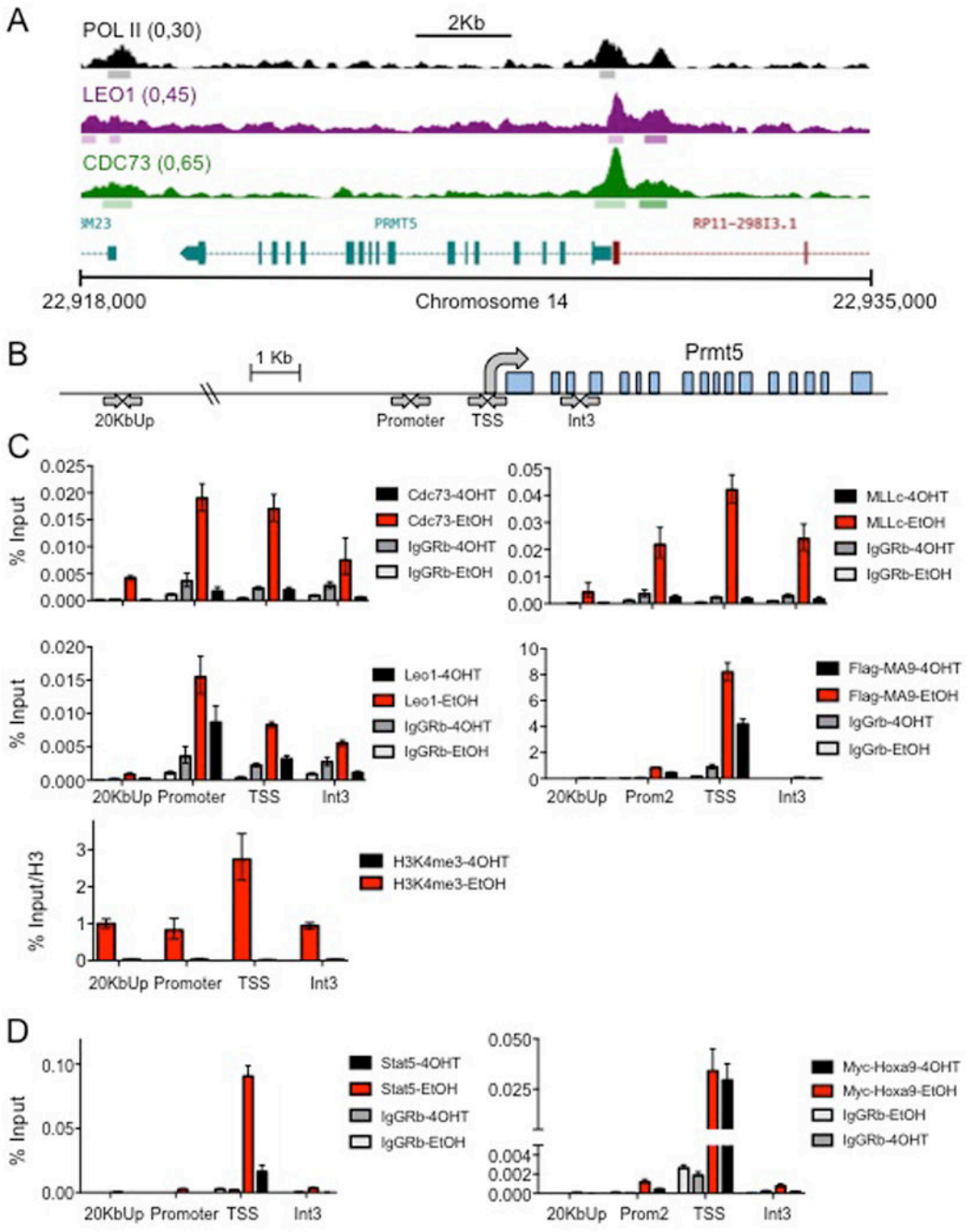


Figure 5. PAFc is a Transcriptional Regulator of *Prmt5* Locus

A) ChIP-sequencing tracks at the PRMT5 locus in human THP-1 leukemic cells[40]. Tracks for RNAPol II, CDC73, and LEO1 are shown. B) Scheme showing the locations on the *Prmt5* locus that were tested by ChIP-qPCR. C) PAFc and MLL-fusion localize to the *Prmt5* locus. MA9-*Cdc73*fl-CreER cells were treated with either 7.5nM EtOH or 4OHT and grown for 48 hours. ~30×10^6 cells were harvested and chromatin immunoprecipitations were performed at the promoter, transcriptional start site (TSS), and Intron 3 of the *Prmt5* locus for CDC73, LEO1, Flag tagged MLL-AF9 fusion, MLLc, and H3K4me3. A site 20kb

upstream of *Prmt5* Locus was used as a negative control. All values are normalized to input or H3 and IgG was used as a non-specific binding control. D) Stat5 and Hoxa9 localize to *Prmt5* locus. Hoxa9/Meis1-*Cdc73*fl-CreERT2 cells were treated as described in (C). Stat5-Y694 and Myc-Tag-71D10 antibodies were used to detect Stat5 and Hoxa9 respectively. ChIP samples performed in biological duplicate and technical triplicate for qPCR.

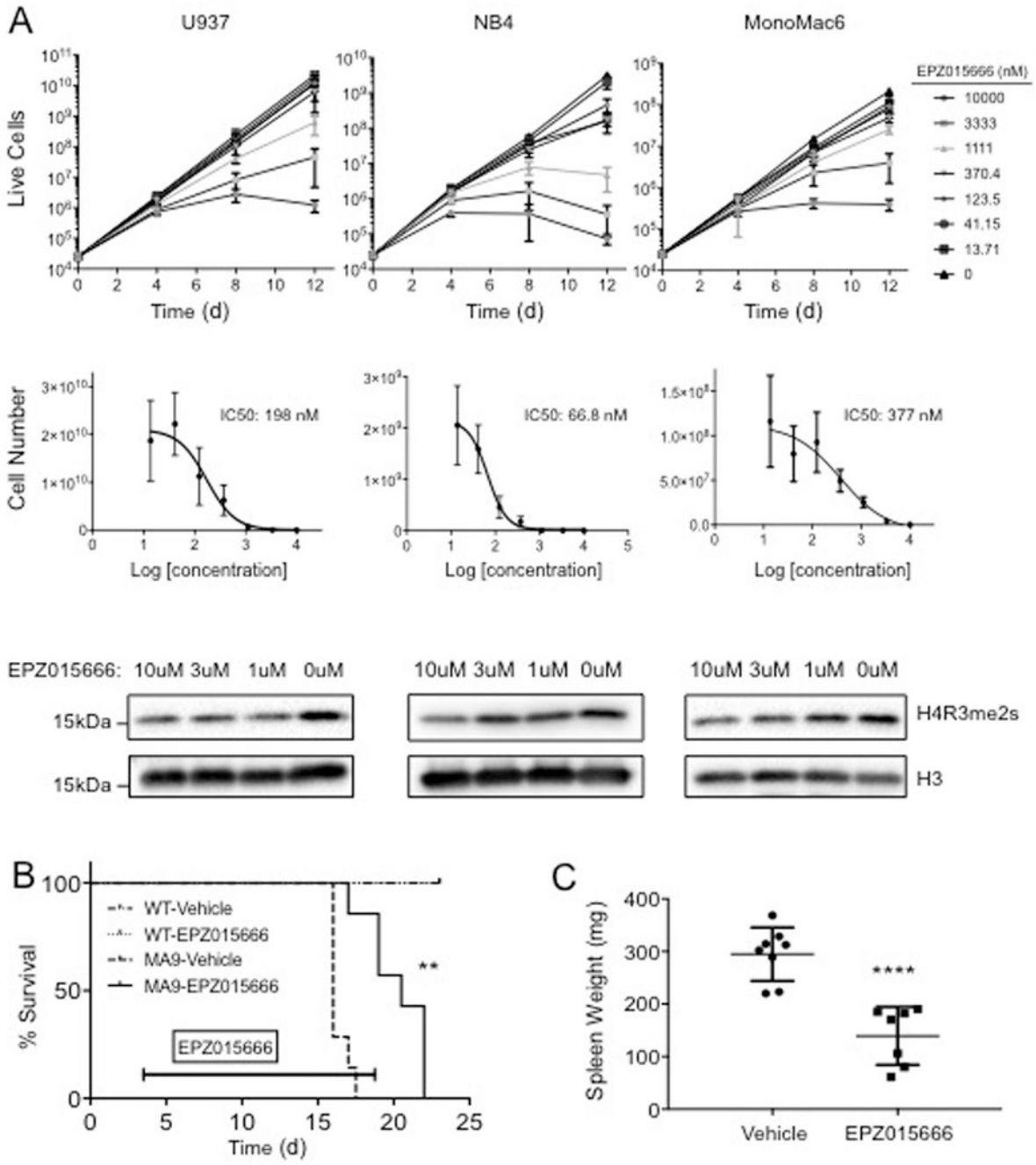


Figure 6. Chemical Inhibition of PRMT5 Reduces Leukemic Growth and Extends Disease Latency

A) Human leukemic cell lines show reduced proliferation upon treatment with PRMT5 inhibitor EPZ015666. U937 (CALM-AF10), NB4 (PML-RARα), and MonoMac6 (MLL-AF9) cell lines were dosed with EPZ015666 at indicated concentrations. Cells numbers were normalized, reseeded, and redosed every 4 days over a 12-day time course. Cell number represents total cells accumulated drawn from the rate of growth between each reseeding. IC50 values were calculated at Day 12 and H4R3me2s is shown at Day 4 for the 10, 3, 1, and 0μM doses. Performed in biological triplicate and technical triplicate. B) Chemical

inhibition of PRMT5 in vivo extends disease latency in a MLL-AF9 mouse model. Sublethally irradiated mice were intravenously injected with 100K primary MLL-AF9 cells (N=7 per dose group) or WT BM cells (N=4 per dose group). At Day 4 mice were subjected to vehicle or EPZ015666 treatment (200mg/kg) BID for 14 days by oral gavage (200μL). ** = p-value < 0.01 by Wilcoxon test. C) Mice dosed with Prmt5 Inhibitor have reduced spleen weight at moribund stage. Spleens were harvested from leukemic mice photographed and weighed. **** = p-value< 0.0001 by student's t-test.